TECHNICAL REPORT
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DISCLAIMER

ABSTRACT

Under this USDOE-NETL contract, the bacterium *Pseudomonas fluorescens* is being developed as a biocontrol agent for zebra mussels *(Dreissena polymorpha* and *Dreissena bugensis*) that infest water pipes in power plants. In the last six months of this project, advances were made in laboratory research concerning the development of a chemically-defined culturing medium for the bacterium. In particular, the following promising medium components were identified: 1) a nitrogen source for use in the basal medium, and 2) amino acids and minerals that had significantly positive effects on cell toxicity and/or yield. A small-scale pipe treatment trial, scheduled to be carried out this past summer in a coal-fired power plant, had to be postponed primarily because of the unexpected difficulty that arose in developing a treatment protocol to achieve high kill of *D. bugensis* – the predominant zebra mussel species at the power plant. The project's schedule has now been delayed a minimum of six months in order to allow additional research to devise an effective treatment protocol for this latter zebra mussel species. How the bacteria are cultured may play a critical role in resolving this *D. bugensis* problem. Preliminary laboratory trials using bacteria grown in our experimental basal culturing medium have produced high *D. bugensis* mortality. This medium, however, will take an additional year to develop before it can be used for culturing large quantities of bacteria in industrial-sized fermentation units. Such bacterial mass production is an essential requirement in order to perform pipe treatment experiments within a power plant.

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EXECUTIVE SUMMARY

Use of the bacterium *Pseudomonas fluorescens* strain CL0145A represents a potential alternative to the current use of polluting biocides for control of zebra mussel infestations in water pipes. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent. This is to be achieved by developing economical methods to culture and formulate the bacterial cells such that they will prove to have good product shelf-life, be environmentally safe, and be highly effective in

killing zebra mussels in power plant water pipes. During the last six months, research efforts focused on the following two activities:

1. Develop a culturing protocol to increase the toxicity of each bacterial cell:

The more toxic each *P. fluorescens* cell is, the fewer cells that will be required in an actual zebra mussel treatment. The need to use fewer cells should consequently result in lower treatment costs. During this six-month reporting period, progress was achieved in laboratory experiments to define the key nutrients and culture conditions required for growth and toxicity of bacterial cells. In particular, the following were identified: 1) a promising nitrogen source for use in a basal medium, and 2) amino acids and minerals that had significantly positive effects on cell toxicity and/or yield.

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2. Conduct a small-scale trial in power plant to demonstrate efficacy:

This three-year project includes annual trials to demonstrate bacterial efficacy in pipes within a coal-fired plant, specifically the Russell Power Station owned by Rochester Gas & Electric in western New York State. These trials were scheduled to begin this past summer with a small-scale trial, then progress to a medium-scale trial next year, and finally culminate in a full-scale treatment of the entire service water system in the third and final year of the project. The trial scheduled to be carried out this past summer, however, had to be postponed, primarily because of the unexpected difficulty that arose in developing a treatment protocol to achieve high kill of *D. bugensis.* This mussel species is one of the two species of zebra mussels that have invaded North America and is the predominant one at the Russell Power Station. The project's schedule has now been delayed a minimum of six months since efforts must now be redirected to devise an effective treatment protocol for this zebra mussel species.

Solving the problem of low *D. bugensis* kill will be facilitated by the progress being currently achieved in our culturing research. A recent laboratory experiment using cells produced from our newly developed basal culturing medium achieved 99% and 100% kill, respectively in *D. bugensis* and *D. polymorpha*. Unfortunately this basal medium, although highly toxic, will first need to be supplemented with other components to increase yield before ever being used in industrial-sized fermentation units for bacterial mass production – a research process that will likely take another year of effort.

INTRODUCTION

Coal-fired power plants within North America need an effective, economical, and non-polluting technique for managing infestations of zebra mussels within their facilities. Due to a lack of options, many facilities have relied on the use of broad-spectrum, chemical biocides for control of these freshwater mussels. However, biocide treatments, such as continuous chlorination for three weeks, are widely regarded as environmentally unacceptable because they can result in the formation of potentially carcinogenic substances. Use of the bacterium *Pseudomonas fluorescens* strain CL0145A represents a potential alternative to the use of polluting biocide treatments and is the leading candidate in the world for the biological control of these macrofouling mussels. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent. This is to be achieved by developing economical methods to produce and formulate the bacterial cells such that they will prove to have good product shelf-life, be environmentally safe, and be highly effective in killing zebra mussels in power plant water pipes. During the six-month period reported herein, research efforts focused on the following two project activities:

1. Develop a culturing protocol to increase the toxicity of each bacterial cell.
2. Conduct a small-scale trial in a power plant to demonstrate efficacy.

EXPERIMENTAL

1. Develop a culturing protocol to increase the toxicity of each bacterial cell:

The following is a general outline of the methodology employed in these culturing tests.

- Shaken seed cultures: One 250-ml Erlenmeyer flask containing 25 ml of buffered tryptic soy broth (bTSB) was inoculated with 0.4 ml of stock culture and shaken at 200 rpm at 26ºC for 24 hr.
- Shaken flask cultures: 0.25 ml from the bTSB 24-hr shaken seed culture was used to inoculate each of 3 replicate flasks containing each experimental medium type. Flasks were shaken at 200 rpm at 26ºC for 24 hr.
- Evaluation of growth of cultures: Growth of cultures was assessed by measuring optical density of final whole culture (FWC) (A_{660} with a Genesys 20 spectrophotometer) in each flask after 24 hr of growth, using the uncultured media of each type as blanks.
- Evaluation of pH of cultures: The pH of media during their preparation and at 24-hr FWC was measured using a pH probe (Corning #476346). The initial pH was determined by measuring the pH of uncultured media of each type (i.e., the blank).
- Production of cell fraction: The final whole culture (FWC) from each flask was centrifuged separately to produce individual pellets from each flask as true replicates. FWC were centrifuged (20 min at 3400 x g) in 25-ml batches in 50 ml centrifuge tubes, and cell pellets were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \bullet 6H_2O$ in deionized water).
- Preparation of cell fraction: The optical density of the cell fraction (CF) inoculum was determined by taking two absorbance readings from the CF at λ = 660 nm at a dilution that resulted in a reading of less than 0.5. The optical density of the CF was then used to calculate the volume of CF required to treat each of 3 replicate micro-chambers (3 replicate micro-chambers treated per each replicate flask) at the target concentration. Mean dry bacterial cell mass/ml for CF were calculated from 2-1.0 ml desiccated subsamples using a Denver Instruments balance. The targeted treatment concentration was 10 or 25 ppm (dry bacterial mass/water volume).
- Preparation of zebra mussels: *D. polymorpha* were collected from the Mohawk River near Crescent, NY, brought back to the lab, sieved, and kept at 7ºC in unchlorinated tap water with filtration and aeration. Approximately 1 week before the test, mussels were moved from 7ºC and acclimated to 23ºC in a 5-gal aquarium containing unchlorinated tap water with aeration (aquarium wrapped in towels to slow warming over several days). The day before the test, 20 <6 mm mussels were placed into micro-chambers containing ca. 5 ml of aerated hard water (Peltier and Weber 1985) and allowed to attach overnight. The morning of the test, unattached mussels were removed and replaced with attached mussels from an extra dish and the water was replaced with 10 ml (micro-chambers) aerated hard water.
- Treatment of zebra mussels with cell fraction: At least one hour before treatment, the micro-chambers were set up with aeration, labeled, and treated (Figs. 1 and 2). After the mussels were exposed for the treatment period (24 hr), the fluid was poured off and mussels were transferred into clean plastic dishes with oxygenated hard water. Mortality was scored and the mussels were held in the dishes for an additional 6 days, changing the water and scoring mortality each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf 1995).

Figure 1: Three micro-chambers each with 10 ml of water and 20 mussels within a plastic container. Each micro-chamber had a single airline with a cotton-filter to maintain aerated conditions throughout the test.

Figure 2: A row of color-coded, plastic containers each holding three micro-chambers. These testing chambers have proven to produce reliable, accurate data, and their small size allows relatively quick set-up and breakdown during an experiment.

2. Conduct a small-scale trial in power plant to demonstrate efficacy:

Preparation for the power plant test required laboratory trials against the two species of zebra mussels present in North America: *D. polymorpha* and *D. bugensis* (the latter species sometimes is also referred to as the "quagga mussel"). The following general procedures were used:

Standard jar testing protocol

- Bacterial culturing:
  - Shaken seed cultures: Two 250-ml Erlenmeyer flasks containing 25 ml of buffered tryptic soy broth (bTSB) were inoculated with 0.4 ml of stock culture and shaken at 200 rpm at 26ºC for 24 hr.
  - Shaken flask cultures: 1 ml from the bTSB 24-hr shaken seed culture was used to inoculate each of 15 replicate flasks containing bTSB. Flasks were grown statically at 26ºC for 72 hr.
  - Production of cell fraction: The final whole culture (FWC) from each flask was pooled and centrifuged (30 min at 1449 x g) to produce a single bacterial pellet. The bacterial pellet was resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \bullet 6H_2O$ in deionized water), thus forming the final cell fraction.
  - Preparation of cell fraction: The optical density of the cell fraction (CF) inoculum was determined by taking two absorbance readings from the CF at λ = 660 nm at a dilution that resulted in a reading less than 0.5. Mean dry bacterial cell mass/ml for CF were calculated from 2-1.0 ml desiccated subsamples using a Denver Instruments balance. The mean dry bacterial cell mass/ml of the CF was then used to calculate the volume of CF required to treat each of the treatment jars at a target concentration of 100 ppm.
- Zebra mussel treatment:
  - Mussels were placed in 975-ml testing jars containing 100 ml of water the day prior to treatment. The morning of treatment, unattached mussels were replaced with attached mussels from a spare jar, the testing jars were filled with 495 ml of water, and their airstones were set at gentle aeration (Fig. 3).
  - Jars were treated with appropriate quantities of bacterial inoculum and held under aerated conditions for 24 hr. Water was then poured off and mussels were placed into clean plastic dishes containing oxygenated hard water. On a daily basis, dead mussels were removed and the water was changed in these dishes.

Figure 3: Line of jars containing mussels being aerated during bacterial exposure.

3. The testing the efficacy of *Pseudomonas fluorescens* against Glenwood Lake *Dreissena* spp.

Tests involving comparison of *D. polymorpha and D. bugensis* populations from Glenwood Lake followed the following general procedure:

- Field collection of specimens:
  Mussels were collected from Glenwood Lake (Medina, NY) by individually pulling mussels from attached surfaces with forceps. Care was taken to pull mussels by their byssal threads so as to not cause internal injury. Jars containing mussels were placed into coolers filled with water from the collection site and aerated until arrival at the Cambridge laboratory where they were placed into flow-through tanks containing natural stream water.
- Feeding treatment scheme:
  Mussels from the collection sites were treated in jars at 100 ppm with bacterial cells stained with safranin and dissected at 1.5 and 3.25 hours to determine feeding behavior on *P. fluorescens.*
- Efficacy treatment scheme:
  Laboratory testing of mussels from the collection sites was conducted at 100 ppm for 24 hours in stream water. The treatment was replicated in a duplicate test to confirm the results.

RESULTS AND DISCUSSION

1. DEVELOP A CULTURING PROTOCOL TO INCREASE THE TOXICITY OF EACH BACTERIAL CELL:

In our previous technical report (41909R01), we indicated that: 1) numerous nitrogen sources had been identified that increased the toxicity of cells harvested from cultures in the chemically semi-defined medium that we have been developing; and 2) future tests would be designed to examine interactions between nitrogen sources over a range of concentrations in order to select possible combinations of nitrogen sources and their most effective concentrations to achieve the highest toxicity. Our testing over the past six months continued on this track by testing the most promising nitrogen sources in a series of factorial experimental designs to determine which nitrogen source(s) were the most effective and which concentration(s) to use in the design of our chemically semi-defined basal medium. This newly designed medium was then used as the base to which other medium components were added alone or in combination in subsequent experiments. Nineteen amino acids were tested in combination in the basal medium using a Plackett-Burman experimental design to evaluate the individual amino acids most important for the expression of toxicity in cultures (Kisaalita et al. 1993). Other components that were

tested included purines and pyrimidines, as well as numerous vitamins and minerals (Zabriske et al. 1980). The analysis of the effects of these components on the toxicity of cultured CL0145A cells will guide the construction of a culture medium that produces cells of significantly higher toxicity than our current complex (chemically-undefined) fermentation medium (FM+).

Nitrogen sources:

Results from the previous six-month period lead to the selection of four nitrogen sources that showed the most promise as components in our medium. Using factorial designs and analyses we were able to assess the individual and combined effects of each of these nitrogen sources. Starting with a 2^4 factorial design (four nitrogen sources each tested at two concentrations) (Table 1), then a 3^2 factorial design (2 variables at 3 levels) (Table 2), we were able to determine the most effective concentration of nitrogen source #1 then determined that nitrogen source #2 did not appear to contribute significantly to increasing toxicity of the cells (Table 3). Therefore, these factorial analyses indicated that nitrogen source #1 at 0.5 g/L was an effective defined nitrogen source in our basal medium.

Table 1: Mean relative toxicity achieved from each of 9 media from a 2^4 factorial design. Results are the combined from two replicate tests.

Medium	Nitrogen source level*				Mean relative toxicity** (2 runs)
	#1	#2	#3	#4	
A	-1	-1	-1	-1	1.67±0.50
B	1	-1	-1	1	1.54±0.23
C	-1	1	-1	1	1.47±0.47
D	1	1	-1	-1	1.55±0.64
E	-1	-1	1	1	1.43±0.44
F	1	-1	1	-1	0.98±0.37
G	-1	1	1	-1	1.00±0.42
H	1	1	1	1	0.99±0.45
Midpoint	0	0	0	0	0.90±0.06

Table 3 footnotes also apply to Table 1.

Table 2: Mean relative toxicity achieved from each of 9 media in a 3^2 factorial design.

Medium	Nitrogen source and level		Relative toxicity	
	#1	#4	25 ppm	10 ppm
A	1	1	1.11	3.99
B	1	2	1.06	3.77
C	1	3	1.11	3.99
D	2	1	1.19	4.39
E	2	2	1.01	3.56
F	2	3	1.18	4.16
G	3	1	1.00	4.36
H	3	2	1.24	5.08
I	3	3	1.20	4.68

Table 3 footnotes also apply to Table 2.

Table 3: Mean relative toxicity achieved from each of 4 media with only nitrogen source #4 varying in concentration.

Medium	Nitrogen source and level		Mean relative toxicity** (2 runs)
	#1	#4	
A	1	0	5.40±2.47
B	1	1	4.42±2.15
C	1	2	5.06±1.96
D	1	3	6.22±2.38

* -1 represents low level, 1 represents high level, and 0 represents a level ½ way between levels -1 and 1.

** Relative toxicities at two target concentrations were calculated as the ratio of mortality from the experimental medium (A through I) compared to the original fermentation medium (FM+).

Amino acids:

Nineteen amino acids were tested in a Plackett-Burman experimental design (Table 4) to test the effects of each amino acid on the growth and toxicity of CL0145A cultures. Statistical analysis of the optical density data indicated that two amino acids had significantly positive effects on growth (Table 5, $p<0.05$). Analysis of the mortality data indicates that two amino acids produced negative effects and three amino acids produced positive effects on cell toxicity to zebra mussels (Table 5, $p<0.05$).

Table 4: Plackett-Burman factorial design developed and analyzed using the Minitab 14 statistical design package. Each medium was tested in triplicate over 6 blocks of tests with the base (w/o) and midpoint included in each block.

Medium	Amino acid																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
A	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1
B	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1
C	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1
D	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1
E	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1
F	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1
G	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1
H	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1
I	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1
J	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1
K	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1
L	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1
M	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1
N	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1
O	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1
P	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1
Q	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1
R	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1
S	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1
w/o	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Midpoint	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5: Factorial analysis: Estimated effects and coefficients for growth (optical density of final whole cultures) and mussel mortality.

Amino acid	Growth (OD of FWC)		Toxicity (mussel mortality)	
	Effect	P	Effect	P
1	-0.06835	0.258	-0.04486	0.443
2	-0.03302	0.584	0.01134	0.846
3	0.08165	0.178	0.03467	0.553
4	0.03831	0.525	**-0.15020**	**0.012**
5	-0.09721	0.110	-0.07526	0.200
6	0.00345	0.954	**0.18447**	**0.002**
7	**0.14631**	**0.017**	**0.19834**	**0.001**
8	-0.07435	0.219	0.10767	0.068
9	0.04345	0.471	0.02240	0.701
10	0.07031	0.245	**0.18894**	**0.002**
11	0.05231	0.386	0.10440	0.077
12	**0.13145**	**0.032**	0.03027	0.605
13	-0.00321	0.957	-0.00086	0.988
14	-0.05521	0.361	**-0.12600**	**0.034**
15	-0.09121	0.133	-0.07200	0.220
16	-0.01235	0.837	-0.03973	0.497
17	0.02279	0.705	0.00354	0.952
18	0.09898	0.103	-0.06586	0.261
19	-0.00655	0.913	-0.08046	0.171

Purines and pyrimidines:

Purines and pyrimidines were tested at three concentrations in the basal medium as a mixture. If the mixture of nucleotides proved to be beneficial to growth or toxicity, then subsequent tests would test the effects of the individual components. However, no significant effects were detected on growth or toxicity when nucleotides were added to the basal medium (Figs. 4 and 5).

Figure 4: Growth of cultures in basal medium containing a range of concentrations of nucleotides.

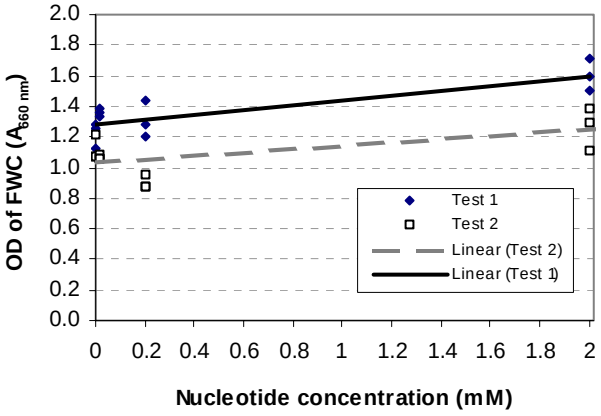
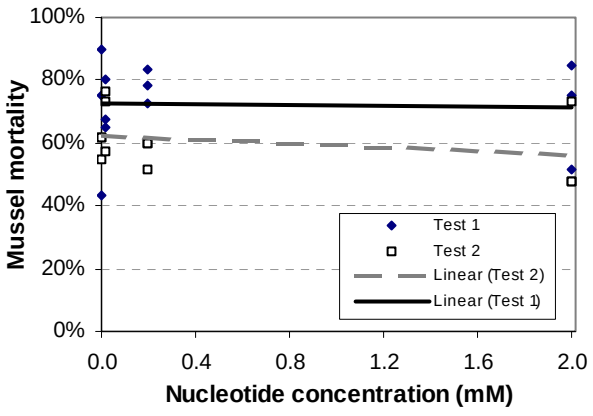


Figure 5: Toxicity of cells harvested from cultures in basal medium containing a range of nucleotides.



Vitamins:

Three concentrations of a mixture containing nine different vitamins were tested in the basal medium. If significant effects were detected on culture growth or toxicity from the presence of this mixture, then individual vitamins would be tested to select the most important individual components. However, the presence of the vitamin mixture in the basal medium did not appear to significantly affect the culture growth or toxicity in these tests (Figs. 6 and 7).

Figure 6: Growth of cultures in basal medium containing a range of concentrations of nucleotides.

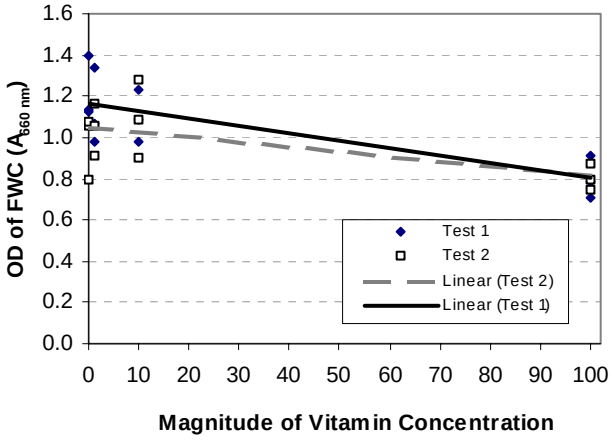
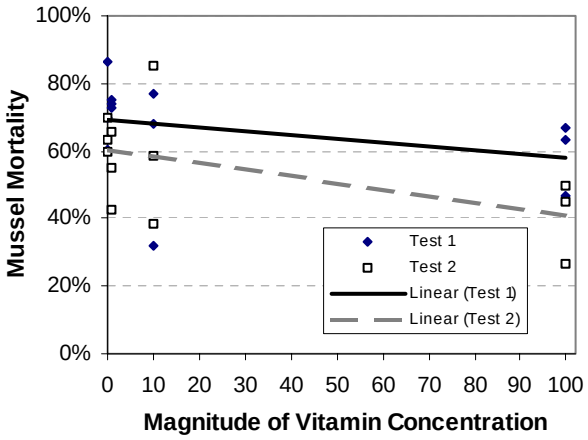


Figure 7: Toxicity of cells harvested from cultures in basal medium containing a range of nucleotides.



Minerals:

Single-addition experiments in which three different minerals were added to the basal medium before culturing identified significant effects from mineral sources. The medium in Figures 8 and 9 designated as “no minerals” is the basal medium to which no minerals were added. Cultures containing mineral #1 grew to an OD approximately three times higher than the basal medium, but less than half as dense as the complex medium, FM+ (Figure 8). The basal medium produced cells that produced seven to ten times higher toxicity than cells from FM+ (Figure 9) and the addition of minerals #1 or #3 did not appear to inhibit or improve toxicity. Therefore, mineral #1 shows promise as a potential additive to the culture medium to increase the density of growth in the cultures without reducing cell toxicity.

Figure 8: Growth of cultures in FM+ and basal medium containing three different minerals.

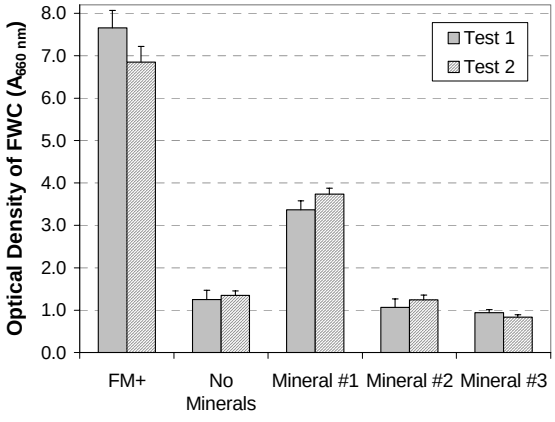
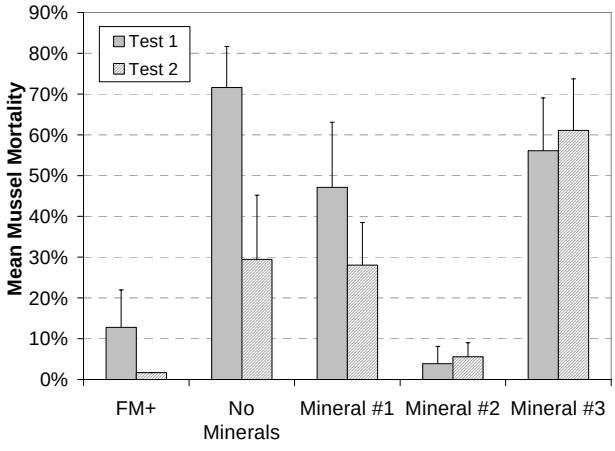


Figure 9: Toxicity of cells harvested from cultures in FM+ and basal medium containing three different minerals.



Basal medium:

The basal medium that has been developed in this period has demonstrated the success of our medium improvement approach for increasing the toxicity of CL0145A cells. The mean culture growth and toxicity data from eight separate tests in which CL0145A was cultured in FM+ and basal medium are presented in Figures 10 and 11. The process of medium component selection to design a defined medium, has led to the development of our current semi-defined basal medium which produced cells achieving 68.3±19.3% mean mussel mortality compared to 7.6±6.8% from FM+ under similar treatment conditions (target concentrations of 10 ppm for 24 hr in micro-chambers) (Fig. 11). Growth of cultures in the basal medium, however, is much lower than in FM+. Mean optical density across these eight tests was 7.34±0.40 and 1.31±0.25 in FM+ and the basal medium, respectively (Fig. 10). At this point in the medium improvement process our focus has been to increase cell toxicity by identifying those medium components that significantly affect the toxicity of cultured cells and, therefore, we have not focused on increasing biomass production. Although we have gained information through these tests on components that increase growth of cultures, such as Mineral #1 (Fig. 8), biomass production will be explored later in the project when the knowledge gained from the defined medium component testing helps to direct our selection of complex medium components, such as hydrolyzed peptones, by predicting which complex components are the most likely to maintain toxicity levels while increasing biomass production.

Figure 10: Growth of CL0145A cultures in FM+ and the basal medium represented as the mean OD from 24-hr shake flask cultures combined from eight tests ($A_{660\ nm}$).

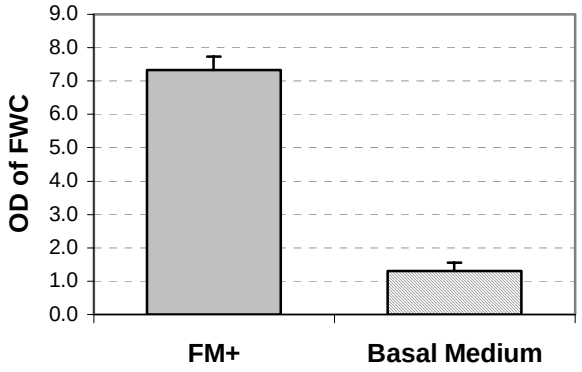
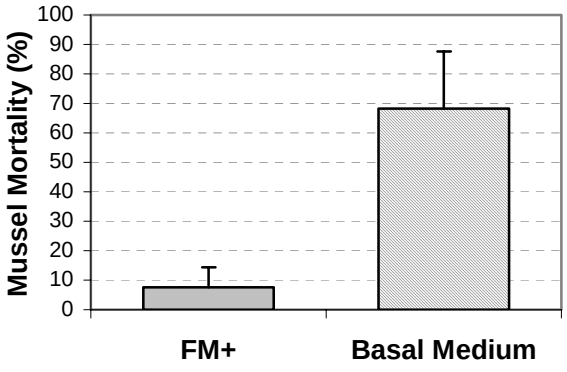


Figure 11: Toxicity of cells harvested from 24-hr shake flask cultures in FM+ and the basal medium taken as the mean combined from eight tests. Mussels were exposed to initial treatment concentrations of 10 ppm for 24 hr in micro-chambers.



Current testing:

The toxicity of CL0145A cells will continue to be improved by conducting tests that will examine the effects of additional components on the efficacy of harvested cells. Continued testing is planned to examine the effects of several minerals on cell toxicity and to repeat the amino acid Plackett-Burman designed tests. If we can identify the amino acids that produce significant positive and negative effects on cell toxicity, the desired amino acid make-up for strain CL0145A can be matched against the amino acid composition of complex peptone sources in the process of selection. Therefore, the amino acid results will be extremely useful in aiding in the selection of complex hydrolyzed peptone sources later in this project. In addition, some of the most effective carbohydrate sources that were tested prior to the selection of nitrogen sources in our current basal medium will be retested in the basal medium. The literature is rich with examples of media designs where a single component is discovered that greatly increases the levels of a given product in culture. Our aim is to continue to screen such components in our defined medium to discover those components that optimize the current levels of toxicity produced from strain CL0145A.

2. CONDUCT A SMALL-SCALE TRIAL IN POWER PLANT TO DEMONSTRATE EFFICACY:

The pipe trial scheduled to occur at the Russell Power Station during this past summer had to be postponed. Following a comprehensive series of laboratory trials, it was judged that bacterial treatment would have produced unacceptably low mussel kill and that it was more prudent to postpone the experiment. With hundreds of hours of effort already invested working at the power plant preparing for the trial, we realized that the likelihood of achieving the desired high kill was low. Considering the major financial investment that would have been required to execute the week-long experiment (e.g., manpower, cost of bacterial inoculum production, 500 mile-round trip travel for the research team), we decided to delay the treatment until a new treatment protocol could be developed, one which would result in the routine achievement of high mussel kill.

Why was low mussel kill anticipated?

Of the two species of zebra mussels in North America, *D. polymorpha* and *D. bugensis* (Fig. 12), *D. polymorpha* is by far the more widely distributed (Fig. 13). We had used that species almost exclusively in our laboratory experiments since the inception of this project in the 1990's. Using prior DOE-NETL funding, for example, we had successfully developed a protocol to treat power plant service water and then successfully used this protocol in the summer of 2003 to consistently kill >90% of *D. polymorpha* in pipe trials within a hydropower plant. The coal-fired plant in Rochester, however, is almost exclusively infested by *D. bugensis*, but we believed from prior experimentation in 2001 that our bacterial treatment of a pipe would be just as likely to kill *D. bugensis* as *D. polymorpha*. To our surprise, however, recent laboratory trials conducted in preparation for this Rochester power plant test, achieved much lower kill for *D. bugensis* than *D. polymorpha*. This was a major unanticipated research development. Thus, it was primarily the low expectations for *D. bugensis* mortality (estimated to be in the 10-60% range) that caused the postponement of the planned service water pipe test. Other factors, such as the detection of chlorine leaking from the power plant's cooling water system into our service water test pipe also became a complicating factor for test planning. This latter problem could have been solved, however, by requesting power plant personnel to temporarily halt their chlorination of the cooling water, but we chose not to do that since the relatively low *D. bugensis* kill expected in the planned trial was enough to postpone the experiment.

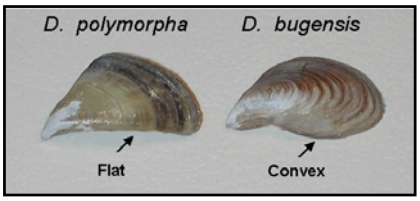


Figure. 12: Differing morphologies of the two *Dreissena* spp. in North America..

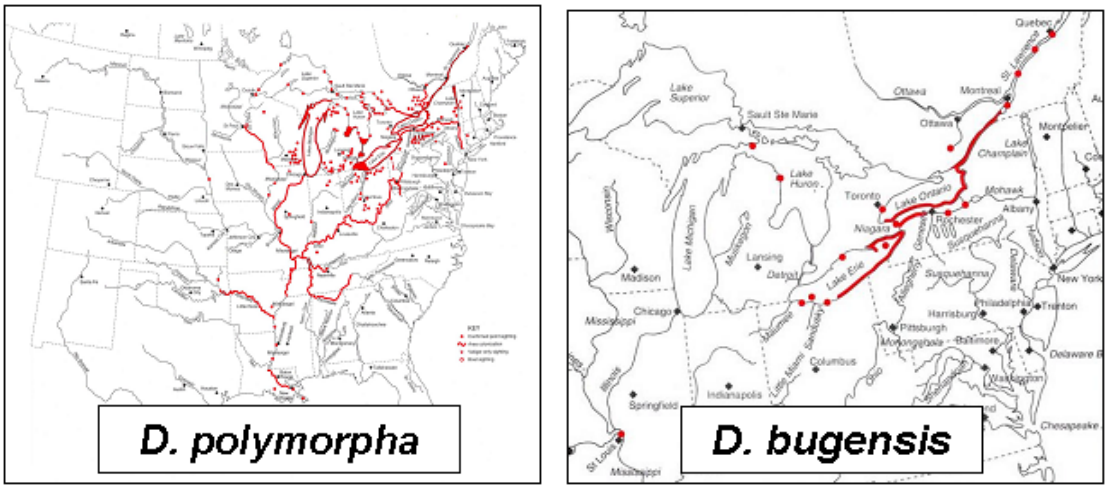


Figure 13: Geographical distributions of the two *Dreissena* spp. in North America.

Why had we thought that we could achieve equal kill with both mussel species?

It is indicated above that our experimentation to date had used *D. polymorpha* almost exclusively as the test species. This was because *D. bugensis* was not locally available for collection and routine testing (closest population was 5 hr away). In our previous DOE-NETL-funded project (DE-FC26-00NT40751), however, we did conduct two tests in 2001 to examine the question of whether equal kill could be achieved with both species. In these latter tests, mussels were collected from two waterbodies where both species coexisted (i.e., Lake Erie and Lake Ontario). The results of these tests clearly suggested that equal kill could be achieved with both species, and this finding was reported in 2001 in DOE-NETL Technical Quarterly Report 40751R02. We were able to get these same results (e.g., approximately 95% kill for both species) this year also, but only rarely. The recent mortality data that we generated were typically in contrast to the 2001 test results, and thus we realized that there were test conditions that existed in 2001 which are not being routinely duplicated in our current 2004 tests.

Do our results indicate that *D. bugensis* is less susceptible to kill than *D. polymorpha*?

No, it would be premature to simply label *D. bugensis* as "less susceptible." That is clear from our 2001 tests in which equally high mortality was uniformly achieved with both species in two separate tests. It is fair, however, to say that *D. bugensis* is less susceptible to kill using our current treatment approach. Following the treatment protocol that we use for killing *D. polymorpha*, we occasionally get 95% *D. bugensis* kill, but it is rare and unpredictable. It is clear that we are not controlling and/or monitoring all experimental variables that are significant in achieving *D. bugensis* mortality. We have also laboratory tested *D. bugensis* and *D. polymorpha* populations collected from Glenwood Lake (NY). Using our standard treatment protocol, we have again found that, *D. bugensis* had less mortality and less feeding on bacteria than *D. polymorpha* collected from the same waterbody (Table 6).

Table 6: Mortality in *Dreissena* spp. from Glenwood Lake*

Species	Relative feeding on bacteria**	Mean % mortality (± SD) Test 1	Mean % mortality (± SD) Test 2	Overall mean % mortality (± SD)
D. bugensis	Low	14.0 ± 6.0	18.7 ± 5.8	16.4 ± 3.3
D. polymorpha	High	50.0 ± 6.9	54.0 ± 8.0	52.0 ± 2.8

* Control mortality < 4.0%

** As determined by dissection and examination of gut contents.

Thus, we have gained evidence that the Rochester power plant population of *D. bugensis* is not an exception, but rather reacts to our standard treatment protocol just like the Glenwood Lake *D. bugensis* population. We thought, based solely on our above-mentioned 2001 tests, that in order to kill *D. bugensis*, we could simply apply the same treatment protocol that was fairly consistent in killing *D. polymorpha*. We have learned that we could not. There are some other "factors," other "variables" (possibly relating to the mussel?, the bacterial inoculum?, environmental conditions?, etc.) that affect whether *D. bugensis* will die following exposure to the bacteria. At present we do not know what they are. That is why we did not want to waste resources attempting to carry out the power plant test, when we realized that we did not know enough about how to treat *D. bugensis* to ensure achievement of high kill.[1]

3. CORRECTIVE PLAN OF ACTION TO ACHIEVE HIGH *D. BUGENSIS* KILL:

What actions need to be taken?

Simply put, we realized that we needed to change our laboratory treatment protocol and adopt one which will result in the routine achievement of high kill for both *D. bugensis* and *D. polymorpha*. Once that is achieved in the laboratory, it can then be tested in actual in-plant pipe trials. We have already initiated this new research plan, and it is a research path that we have successfully gone down before.

[1] More recent laboratory trials using *D. polymorpha* and *D. bugensis* from another New York State waterbody, Seneca Lake, have also confirmed the pattern of lower *D. bugensis* mortality (see Tables 7-11).

The DOE-NETL project that preceded this one (i.e., DE-FC26-00NT40751) had this same objective with *D. polymorpha*. In that project, we carefully evaluated biotic and abiotic factors that influenced *D. polymorpha* kill. From that effort we developed a protocol for pipe treatment – a protocol which when evaluated last year in pipe treatments within a hydropower plant consistently achieved >90% *D. polymorpha* mortality. We are now focusing on doing the same with *D. bugensis*. The down side of this plan is that it will require a major diversion of research effort, causing the project to be set back 6-months at a minimum.

Redirection of research efforts:

As mentioned above, we have found that our standard laboratory testing protocol that routinely killed >90% of *D. polymorpha* produced lower mortalities among *D. bugensis*. However, there have been tests that achieved similarly high kill to *D. bugensis*, suggesting that *D. bugensis* is susceptible to the toxin under certain conditions. Our research efforts thus have been refocused to identify the key variables that will consistently produce high levels of mortality among both *D. polymorpha* and *D. bugensis*.

These redirected research efforts have involved an expanded series of laboratory trials, and this additional testing has required collection of tens of thousands of mussels of both species. Thus, our first objective was to find a waterbody in which high density populations of both *D. polymorpha* and *D. bugensis* co-existed. This could not be from the Rochester area of Lake Ontario near the Russell Power Station since that region contains few *D. polymorpha*. Nor could it be from Glenwood Lake which, although it had high densities of both species, was polluted with sewage from a nearby town. Ideally the waterbody chosen for collecting both species would be similar in water chemistry to the waters flowing through the Russell Power Station. We found such a waterbody, Seneca Lake (Geneva, NY), which like Lake Ontario, is meso-oligotrophic. We were fortunate since Seneca Lake currently contains populations of *D. polymorpha* and *D. bugensis* of high density. As a result, this Seneca Lake population became our standard source for testing.

Our second objective was to define a testing protocol with which consistent mortality results could be achieved among both *D. polymorpha* and *D. bugensis*. In a series of three replicate tests, spanning two separate collections from Seneca Lake in August 2004, we achieved this objective. Modifications to our standard jar testing protocol for *D. polymorpha* produced consistent levels of mortality to *D. bugensis*. These modifications included:

- Reducing disturbances during testing by reducing the overall manipulation and disturbance to the mussels during preparation and treatment of mussels.
- Producing frozen cells of CL0145A from a single batch of cultures to be used in multiple tests. The dry weight of the frozen cell solution was determined prior to treatments to reduce variability in treatment concentrations between tests.
- Mussel siphoning was observed and recorded periodically before, during, and following application of CL0145A treatments to identify differences between *D. polymorpha* and *D. bugensis* behavior and to identify other variables that might impact mussel feeding behavior.
- Mussel feeding was analyzed through dissections of mussels 0.5 to 3.5 hr following cell treatment. Dissected mussels were viewed using a dissection microscope. The presence of bacteria in the stomach, digestive gland, and intestine were recorded in addition to the state of the reproductive organs (i.e., gravid or not). The presence of bacterial cells was confirmed using a compound microscope at 450-1000X magnification.

In these three tests, the resulting mussel mortalities, as anticipated, were much lower among treated *D. bugensis* than *D. polymorpha* (13.7±1.22% and 94.54±3.05%, respectively, Table 7). As hoped for, the results of all three tests were very consistent. The distinct and consistent difference between mortalities of these two Seneca Lake species now can be used advantageously as an experimental tool to detect the effects on mussel mortality of modifying individual variables in subsequent laboratory experiments.

Table 7: Mortality in Seneca Lake *Dreissena* spp. in three replicate tests using the modified laboratory testing protocol. Treatments were 100 ppm (initial treatment concentration) of frozen cells from the same batch of bacterial cultures for 24 hr in testing jars (500 ml hard water).

	Mean mussel mortality in each test			
Species	Test 1	Test 2	Test 3	Mean mortality overall
D. polymorpha	93.33±1.15%	92.27±1.61%	98.00±2.00%	94.54±3.05%
D. bugensis	12.33±2.52%	14.67±3.51%	14.14±3.79%	13.71±1.22%

Testing for key variables:

Once the standard testing protocol was determined, we assessed the affects of individual variables on the efficacy of CL0145A treatments while always including the modified laboratory testing protocol as a standard in each of the tests. In this way, potential affects due to the alteration of a single variable could be compared to the results from the standard treatment method for both *D. polymorpha* and *D. bugensis.* This approach will guide us in attempting to identify the biotic and abiotic factors that result in CL0145A treatments being more or less effective against *D. bugensis.*

- Mussel size:

  A variety of size classes of mussels of both species were treated to determine whether a certain size class would be more susceptible to treatments with fresh CF (cell fraction) at 100 ppm for 24 hr. In these tests, high mortality was achieved among *D. polymorpha* (i.e., 97-100% kill) in all size classes. Lower mortality was achieved among *D. bugensis* (i.e., 36-88% kill), with no clear trend in mortality between small and large mussels (Table 8). In many prior tests with *D. polymorpha*, we have noted the size was not a critical factor in determining the susceptibility of *D. polymorpha*. This also appears to be the case with *D. bugensis*, with this latter conclusion supported by this current testing (Table 8) as well as in *D. bugensis* size trials conducted three years ago (DOE-NETL Technical Quarterly Report 40751R02).

  Table 8: Mortality in Seneca Lake *Dreissena* spp. following standard laboratory treatments to different size classes in two separate tests.

Species	Test	Size class	Mean mussel length (mm ±SD)	Mean mortality (%±SD)
D. polymorpha	Test 1	Small	4.4±1.0 mm	100.0±0.0%
		Large	8.4±1.9 mm	97.3±3.1%
	Test 2	Small	4.5±0.7 mm	100.0±0.0%
		Large	No data*	100.0±0.0%
D. bugensis	Test 1	Small	4.2±1.3 mm	49.8±16.3%
		Large	9.9±1.9 mm	61.3±11.7%
	Test 2	Small	4.5±1.0 mm	88.0±3.5%
		Large	8.9±2.7 mm	36.8±15.0%

* Mussel lengths were not measured, but were estimated to be ca. 8-10 mm.

- Treatment regime:

  D. bugensis mussel populations are known to be successful in low nutrient (i.e., oligotrophic) waterbodies while *D. polymorpha* populations appear to prefer more nutrified (i.e., eutrophic) environments although this pattern is not exclusive (Jarvis et al. 2000, Petrie and Knapton 1999, Roe and MacIsaac 1997, and Mills et al. 1993). We hypothesized that the relatively low kill observed in *D. bugensis* might be the result of its low nutrient feeding mechanisms becoming overwhelmed by single high treatment concentrations of bacteria and that kill might be enhanced by using lower treatment concentrations. Therefore, a preliminary laboratory test was designed to compare two different treatment regimes in testing jars. In this test, we compared our standard

treatment protocol in which 100 ppm of cells are added to the testing jar at one time *versus* a modified protocol in which 12.5 ppm of cells were added each 0.5 hour until a cumulative treatment of 100 ppm was reached, i.e., 4 hours. In this test there did not appear to be a difference in mussel mortality due to treatment regime in either mussel species (Table 9). However, this was a preliminary test representing only one of many possible treatment regimes to try, which we are planning to test under once-through conditions using laboratory-scale mini-pipes.

Table 9: Mortality in Seneca Lake *Dreissena* spp. following standard laboratory treatments to different size classes in two separate tests.

Species	Treatment	Mean mortality (%±SD)
D. polymorpha	Single 100 ppm	100.0±0.0%
	Cumulative 100 ppm (12.5 ppm x 8)	98.6±1.2%
D. bugensis	Single 100 ppm	72.0±4.0%
	Cumulative 100 ppm (12.5 ppm x 8)	61.3±10.3%

- Cell formulation:

  Previous testing at our laboratory has shown that CL0145A cells maintain their toxicity to *D. polymorpha* for extended periods (i.e., for at least three months) if stored frozen at -80ºC, but we've also observed a change in the consistency of the CF after it has been frozen and then thawed. We tested both species of mussels with fresh and frozen (-80ºC) cells from the same batch of cultures to assess whether this aspect of our current treatment formulation has an effect on the treatments. In this test, frozen and fresh cells were equally toxic to *D. polymorpha* (98.7±2.3% and 100.00±0.0% kill, respectively), but fresh CF treatments achieved higher mortality to *D. bugensis* than did frozen CF treatments (88.0±3.5% and 58.0±8.7% kill, respectively, Table 10). Thus, mortality results from this test suggest that the formulation presented to the mussels may affect the efficacy of CL0145A treatments, particularly to *D. bugensis*. Since one of the tasks of the current grant is to develop an effective formulation of this bacterial strain, developments in formulation could lead to increased effectiveness of CL0145A to *D. bugensis*.

Table 10: Mortality in Seneca Lake *Dreissena* spp. following standard laboratory treatments to frozen and fresh formulations of CL0145A cells.

Species	Treatment	Mean mortality (%±SD)
D. polymorpha	Frozen CF	98.7±2.3%
	Fresh CF	100.0 ±0.0%
D. bugensis	Frozen CF	58.0±8.7%
	Fresh CF	88.0±3.5%

- Culture medium:

  Tests outlined in detail earlier in this report described our progress in the improvement of our culture medium to produce cells that demonstrate significantly higher levels of mussel mortality. The medium improvement bioassay results have demonstrated significantly higher toxicities to *D. polymorpha* from cells harvested from shake-flask cultures grown in our improved semi-defined basal medium compared to cells harvested from our current complex fermentation medium (FM+) or static cultures in bTSB (buffered tryptic soy broth) (Table 11). We tested the effectiveness of treatments with cells harvested from static cultures (i.e., our standard CF), and shake-flask cultures in FM+ and the basal medium on both species of mussels (Table 11). Although cells from all three types of media (bTSB, FM+ and the basal medium) produced high levels of mortality to *D. polymorpha* (>96%), differences were observed in mortality to *D. bugensis*

depending on which type of media the cells were cultured in (Table 11). In two tests, higher mortality among *D. bugensis* was achieved with cells harvested the basal medium (98.7% and 88.7% kill) than with bTSB (54.7% and 66.0% kill) or FM+ (42.7% one test) (Table 11). Therefore, these results suggest that continued increases in CL0145A toxicity through the improvement of the culture medium will correspondingly increase its effectiveness against both species of mussels. This is of particular importance for *D. bugensis*.

Table 11: Mortality in Seneca Lake *Dreissena* spp. after standard laboratory treatments with cells harvested from static cultures in bTSB or shaken cultures in FM+ or basal medium.

Species	Culture description and medium	Actual treatment concentration	Mean mortality (%±SD)
D. polymorpha		TEST #1	
	bTSB static	100 ppm	98.7±1.2%
	FM+ shaken	54 ppm	98.7±1.1%
	Basal medium shaken	82 ppm	100.0±0.0%
		TEST #2	
	bTSB static	25 ppm	98.0±0.0%
		100 ppm	97.3±3.1%
	Basal medium shaken	20 ppm	96.7±3.1%
		78 ppm	99.3±1.2%
D. bugensis		TEST #1	
	bTSB static	100 ppm	54.7±7.6%
	FM+ shaken	54 ppm	42.7±13.3%
	Basal medium shaken	82 ppm	98.7±1.2%
		TEST #2	
	bTSB static	25 ppm	22.0±0.0%
		100 ppm	66.0±8.7%
	Basal medium shaken	20 ppm	68.7±10.3%
		78 ppm	88.7±7.6%

Action plan for increasing efficacy to *D. bugensis*:

Recent treatments of *D. bugensis* have demonstrated that under our standard laboratory treatment conditions, lower kill will be expected to *D. bugensis* than to *D. polymorpha.* However, because high kill (>95%) has been achieved to *D. bugensis* in some tests using our standard treatment protocols, we believe that there are certain testing conditions under which high kill can consistently be achieved to *D. bugensis.* We have designed a plan to identify those currently unknown variables. Some experiments that are being considered in this approach are outlined below:

- Reduce disturbance and light: Mimic conditions predicted to be found in pipes within power plants in laboratory-scale once-through pipes tests, including:
  - Placing mussels in the pipes under flow well before treatment (ca. 1 week) to allow the mussels to acclimate more fully and to reduce the disturbances caused by handling preceding treatment.
  - Eliminate light by enclosing the pipes in light-excluding materials. Mussel behavior (i.e., siphoning) could still be observed using an infrared camera.
- Treatment date: Assess whether the collection date affects mussel susceptibility by treating *D. polymorpha* and *D. bugensis* by collecting mussels from the same population (i.e., Seneca Lake, NY) at different dates over a period of months and assessing reproductive state, ingestion, and mussel mortality among both species.
- Particle ingestion experiments: Treat *D. polymorpha* and *D. bugensis* with various size and types of particles and observe and compare ingestion through the examination of the stomach, digestive gland, and intestine of dissected mussels to gain information on preferred particle type and size for

both species. Some particles to be assessed include stained and unstained beads, bacterial cells, and phytoplankton (i.e., algae ranging in size from ca. 0.1 to 15-μm). If we can determine a preferred particle size for effective ingestion by both species of mussels, we could adjust the particle size of the bacterial cells in future tests in the development of an effective bacterial cell formulation.

- Sensitivity to the toxin: Compare the ingestion of cells of our toxic strain of *P. fluorescens* (strain CL0145A) to cells of a non-toxic strain of *P fluorescens* to assess whether *D. bugensis* is sensitive to the presence of the toxin, causing the mussels to ingest less biomass.
- Presence of naturally-occurring particles: Assess whether the presence of naturally-occurring food (i.e., seston or algae) in the mussels' digestive system affects the efficacy of bacterial treatments. "Feed" mussels algal or seston particles before, during, and after treatment with bacteria and assess mussel mortality.
- Treatment regime: Assess the effect of treatment regime on mussel ingestion and mortality by treating *D. polymorpha* and *D. bugensis* at various bacterial concentrations or in intermittent doses (i.e., pulsed treatments) under once-through conditions. Treatments at lower dosages for longer time periods may be more effective than shorter treatments at higher dosages (i.e., 25 ppm treatments for 24 hr *versus* 100 ppm treatments for 6 hr).
- Velocity: Assess the effect of velocity on treatment efficacy by treating *D. polymorpha* and *D. bugensis* under various levels of flow under once-through conditions in laboratory-scale pipes.
- Oxygenation: Assess the effect of different levels of oxygenation during treatment on efficacy. In laboratory tests with *D. polymorpha,* we have found that treatments appear to be the most effective under highly aerated conditions, but *D. bugensis* may react differently to oxygenation.

Using the described laboratory-scale approach we hope to identify conditions and treatment protocols that consistently produce high levels of mortality to both *D. polymorpha* and *D. bugensis* so that these factors can be applied to treatments in the field (i.e., pipe treatments in the RG&E power plant) to increase the likelihood of success in these trials.

CONCLUSIONS

Significant progress has been made in tests designed to identify a culturing protocol to increase the toxicity of each bacterial cell. Unfortunately these culturing efforts have had to be put on hold since staff working on culturing were reassigned to experiments exploring possible causes of the relatively low kill that has been observed in *D. bugensis* – the predominant zebra mussel species at the Russell Station Power Plant. The project's schedule has now been delayed a minimum of six months in order to allow additional research to devise an effective treatment protocol for this latter zebra mussel species. In this regard, how the bacteria are cultured may play a critical role. Preliminary laboratory trials using bacteria grown in our experimental basal culturing medium have produced high *D. bugensis* mortality. This medium, however, will take an additional year to develop before it can be used for culturing large quantities of bacteria in industrial-sized fermentation units in order to provide enough inoculum to perform a pipe treatment experiment within a power plant.

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TECHNOLOGY AND INFORMATION TRANSFER

This project was highlighted in the following presentations:

Mayer, D. A. Development of *Pseudomonas fluorescens* strain CL0145A as a biological control against zebra mussels (*Dreissena polymorpha*). Department of Biological Sciences of the University at Albany. September 10, 2004. Albany, NY (Invited speaker.)

Mayer, D. A., Molloy, D. P., Morse, J. T., Presti, K. T. Sawyko, P. M., and Sprague, P. A. Biocontrol of zebra mussels: The path to commercialization of *Pseudomonas fluorescens* strain CL0145A. Annual Meeting of the Society for Industrial Microbiology. July 26, 2004. Anaheim, CA (Submitted poster.)

Morse, J. Control of zebra mussels with the biopesticide *Pseudomonas fluorescens*. New York State Department of Environmental Conservation, Avon, NY. May 6, 2004. (Invited speaker.)